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# PLATELET ANGIOGENIC ACTIVITIES: A CONCERT OF MULTIPLE PLAYERS

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# Platelet angiogenic activities: A concert of multiple players

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To myself and my beloved family

# ABSTRACT

Angiogenesis, the sprouting of new capillaries from pre-existing blood vessels, is crucial for many physiological and pathological processes. Platelets are an important player in angiogenesis, whilst understanding of platelet engagement remains obscure. The present thesis aims to further elucidate the mechanisms of platelet angiogenic activities, including those by platelet releasate, platelet membrane, and miRNA-regulated platelet angiogenic factor synthesis.

Our earlier studies have demonstrated that thrombin protease activated receptor 1 (PAR1) and PAR4 stimuli induce selective platelet release of proangiogenic and antiangiogenic regulators, respectively. The present thesis work shows that both PAR1-stimulated platelet releasate (PAR1-PR) and PAR4-PR enhanced tube formation and migration of endothelial progenitor cells (EPCs, also termed as endothelial colony forming cells, ECFCs, in paper II and IV) in vitro and angiogenesis in vivo, while the enhancements by PAR1-PR were more potent than those of PAR4-PR. Our findings suggest that the selective release of platelet angiogenic regulators may mainly concern the different release levels of platelet angiogenic regulators, and the final outcome of angiogenic regulating effects will depend on the negotiation of all factors in the platelet releasates.

We further demonstrated that platelet-promoted angiogenesis depends on not only platelet-released mediators but also platelet membrane components. Thus, we found that platelet-enhanced EPC angiogenic responses were more pronounced than those by platelet releasates. We showed that platelet membrane glycoproteins had a major role in promoting EPC tube formation, and have identified that platelet tetraspanin CD151 and the integrin α6β1 expressed on both platelets and EPCs contributing to platelet-promoted tube formation.

Albeit as anucleated cells, platelets retain certain capacities of protein synthesis. Our work gives further evidence that thrombin stimulation induced de novo synthesis of thrombospondin 1 (TSP1). More importantly, we showed that thrombin stimulation altered platelet miroRNA profile, and that thrombin-reduced platelet miR-27b expression enhanced TSP1 synthesis. The latter was supported by our data showing that miR-27b mimic transfection inhibited TSP1 synthesis in the platelet precursor megakaryocytes.

We have also studied if type 2 diabetes mellitus (T2DM) would impair platelet angiogenic activities. We found that platelets from mild T2DM subjects were hyperreactive, but had similar angiogenic activities (angiogenic regulator release and platelet-regulated EPC tube formation), as compared to those of age/gender-matched controls. Our data suggest that a good glucose control may be beneficial for maintaining platelet angiogenic function.

Together, this thesis work shows the platelets can regulate angiogenesis via different elements, and that platelet angiogenic activities are a concert of multiple players, namely released angiogenic factors, membrane components, as well de novo angiogenic factor synthesis and its regulation by miRNAs.

# LIST OF SCIENTIFIC PAPERS

I. Huang Z, **MIAO X**, Luan Y, Zhu L, Kong F, Lu Q, Pernow J, Nilsson G, Li N. PAR1-stimulated platelet releasate promotes angiogenic activities of endothelial progenitor cells more potently than PAR4-stimulated platelet releasate. J Thromb Haemost 2015; 13:465–76.

II. Huang Z, **MIAO X**, Patarroyo M, Nilsson GP, Pernow J, Li N. Tetraspanin CD151 and integrin a6b1 mediate platelet enhanced endothelial colony forming cell angiogenesis. J Thromb Haemost 2016; 14: 606–18.

III. **MIAO X**, Rahman MF, Jiang L, Xie H, Lui WO, Li N. Thrombin-reduced platelet miR-27b expression enhances de novo synthesis of thrombospondin-1 in platelets. Manuscript.

IV. **MIAO X**, Zhang W, Huang Z, Li N. Unaltered angiogenesis-regulating activities of platelets in mild type 2 diabetes mellitus despite a marked platelet hyperreactivity. PLoS ONE 2016.11(9):e0162405.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-HT2A | Serotonin 2A receptor |
| Ago2 | Argonaute 2 |
| Ang | Angiopoietin |
| bFGF | Basic fibroblast growth factor |
| ECFC | Endothelial colony forming cell |
| ECM | Extracellular matrix |
| EC | Endothelial cell |
| EPC | Endothelial progenitor cell |
| GPIb-IX-V | Glycoprotein Ib-IX-V complex |
| GPIIb/IIIa | Glycoprotein IIb/IIIa complex |
| GPVI | Glycoprotein VI |
| miRNAs | microRNAs |
| MMPs | Matrix metalloproteinases |
| mRNA | messenger RNA |
| MT- MMPs | Membrane-type MMPs |
| PAI-1 | Plasminogen activator inhibitor 1 |
| PAR | Protease-activated receptor |
| PAR1 | Protease activated receptor 1 |
| PAR1-PR | PAR1-stimulated platelet releasate |
| PAR4 | Protease activated receptor 4 |
| PAR4-PR | PAR4-stimulated platelet releasate |
| PDGF | Platelet-derived growth factor |
| PDGF-Rα | Platelet-derived growth factor α receptor |
| PDGF-Rβ | Platelet-derived growth factor β receptor |
| PDMP | Platelet-derived microparticle |
| PF4 | Platelet factor 4 |
| $PGE_1$ | Prostaglandin $E_1$ |
| $PGI_2$ | Prostaglandin $I_2$ |

| | |
|---|---|
| PI | Propidium iodide |
| PlGF | Placental growth factor |
| PRP | Platelet-rich plasma |
| qRT-PCR | Quantitative reverse transcription PCR |
| RBP | RNA binding protein |
| RIP | RNA immunoprecipitation |
| RISC | RNA-induced silencing complex |
| SDF-1 | Stromal cell-derived factor-1 |
| T2DM | Type 2 diabetes mellitus |
| TGFβ | Transforming growth factor β |
| TIMPs | Inhibitors of metalloproteinases |
| TNF | Tumor necrosis factor |
| TSP-1 | Thrombospondin-1 |
| $TXA_2$ | Thromboxane $A_2$ |
| VEGF | Vascular endothelial growth factor |
| vWF | von Willebrand factor |

# 1 INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels. After the establishment of the first vessel network in the developing embryo, angiogenesis is responsible for the most blood vessel formation during development and growth. Angiogenesis, characterized by sprouting and tube formation occurring in the network of endothelial cells (ECs), is a physiological and vital process in growth and development, in wound healing, as well as in the transition of tumors from a benign state to a malignant one[1]. Angiogenesis is a tightly regulated process, and is under the control of both positive and negative regulatory elements, requiring the coordinated actions of a variety of growth factors /receptors, and cell-cell interactions among ECs, other vascular vessel wall cells, and their surrounding microenvironment. Therefore, disturbances in physiological angiogenesis can contribute to various human diseases, including cancer, cardiovascular diseases, diabetic complications, ocular diseases, chronic inflammation and so on[1].

Platelets are small anucleate cells shed from megakaryocytes in the bone marrow, and play an essential role in surveillance of vascular injury after being released into the circulation. Platelets can quickly adhere and aggregate at the site of injury, which is a critical event for hemostasis[2]. In addition to the most well-known function, increasing evidence shows that platelets also play an important role in angiogenesis. The first scientific evidence in 1960s demonstrated that perfusion of the organs with platelet-depleted plasma caused instability of the endothelial layer, parenchymal degeneration, and hemorrhages, and that the addition of platelets markedly reduced this injurious effect[3]. The observation has built the foundation for the development of the current concept of platelet involvement in angiogenesis. After that, the relevance of platelets in angiogenesis has been highlighted by many other publications.

## 1.1 ANGIOGENESIS REGULATION

In recent years, there have been tremendous efforts to uncover the molecular mechanisms that drive blood vessel growth. There are several factors that regulate angiogenesis. Some of these factors act as stimulants to angiogenesis or creation of new blood vessels, whilst others act as inhibitors to hamper or prevent angiogenesis.

### 1.1.1 Triggers of angiogenesis

(1) Vascular endothelial growth factor (VEGF)

VEGF possesses a dominant role in mediating EC sprouting, migration, and network formation, as indicated by the early lethality of VEGF-deficient mice[4]. VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) [5]. Among these, VEGF-A (often referred as VEGF) is the most well-characterised isoform, and plays a key role in the migration and proliferation of ECs.

VEGF transmits the signal through two main receptors with the function of tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1, KDR)[6]. VEGF-A binds VEGFR-1 with higher

affinity than VEGFR-2, but the tyrosine kinase activity of VEGFR-1 is weaker than VEGFR-2. Lack of VEGFR-1 decreases sprout formation and reduced migration, however, a soluble isoform of VEGFR-1 (sVEGFR-1) rescued angiogenesis[7]. Knockout of VEGFR-2 leads to aberrant vascular development and embryonic lethality [8].VEGF-VEGFR2 binding starts a tyrosine kinase signaling cascade that stimulates the production of factors that stimulate vessel permeability, proliferation/survival, migration and finally differentiation into mature blood vessels.

(2) Basic fibroblast growth factor (bFGF)

bFGF is the first idetified proangiogenic factor[9], which has two high affinity receptors (FGF-R1 and FGF-R2). With cultured ECs, bFGF induces an angiogenic phenotype of endothelial cells, which are seen with increased proliferation, migration and proteinase production [10]. Moreover, bFGF also induces the formation of angioblasts from mesenchymal cells [10]. In addition, the use of antisense RNAs against bFGF or FGF-R1 has been shown to induce severe vascular disorders [11, 12].

(3) Angiopoietin (Ang)

Three members of the Ang family have been described: Ang1, Ang2, and Ang3 (in mouse)/4 (in human). They bind to the Tie1/Tie2 endothelial-specific receptors tyrosine kinase. Ang-1 is a single non-redundant agonist of Tie-2 required for ECs survival and proliferation and for vessel maturation [13]. By binding to the receptor Tie-2, Ang-1 stimulates recruitment of pericytes, which cover endothelial sprouts and stabilise them [13]. Mutations in the genes of Ang-1 and Tie-2 cause vessel deformity and early embryonic lethality[14, 15]. Interestingly, a family of ligands, Ang-2, has been identified as an antagonist of Ang-1 in ECs, as evidenced by a similar embryonic phenotype when Ang-2 was overexpressed in transgenic mice.

(4) Platelet-derived growth factor (PDGF)

PDGF exerts its functions by binding to and activating PDGF receptors on the cell membrane[16]. Two different monomers exist, known as A chain and B chain, which assemble into PDGF-AA, PDGF -BB as well as PDGF -AB. PDGF molecules interact differentially with two known PDGF receptors, the α receptor(PDGF-Rα) and the β receptor (PDGF-Rβ). PDGFRα has a high affinity for PDGF-A and -B, whereas PDGFRβ has a high affinity for PDGF-B. Ligand binding to receptors induces receptor dimerization, which leads to activation of the intrinsic tyrosine kinase domain, subsequently activation of these pathways leads to cellular responses, like proliferation and migration. A lot of evidence has been accumulated that supports the contribution of PDGF/PDGFR axis in developing angiogenesis in both normal and tumoral conditions.

Knock-down of PDGFRα, as well as PDGFRβ, by siRNA suppressed growth of prostate cancer cells in mice and tumor angiogenesis [17]. It has also been shown that, using a knockout model, PDGF-B/PDGFRβ signaling is critical in the establishment of functional

blood vessels by recruiting and stabilization of perivascular cells [18]. PDGF-B and PDGFRβ knockout mice die perinatally from vascular defects found in many organs[19, 20]. PDGF also induces angiogenesis by up-regulating VEGF production and modulating the proliferation and recruitment of perivascular cells [21].

(5) Transforming growth factor β (TGFβ)

TGFβ is a pleiotropic factor that plays pivotal roles in both vasculogenesis and angiogenesis. TGFβ drives vascular responses via its binding to TGFβ receptor complex, type I (ALK 1-7) and type II (TGFβRII, BMPRII, ActRIIA, ActRIIB, and MISRII) serine/threonine kinase receptors[22]. For regulation of endothelial function by TGFβ, ALK1 and ALK5 signaling are most important.

Signaling by these receptors is context-dependent and tightly regulated, particularly in cultured ECs, where TGFβ can either promote or suppress endothelial migration, proliferation, permeability, and sprouting[23]. Together with evidence suggesting that TGFβ is a central mediator of angiogenesis, TGFβ may play contrasting roles depending on the stage of the process. An in vitro study has shown that ALK1 antibody inhibited EC sprouting [24]. However, in the mice model of ALK1 deficiency, ALK1 signaling cooperates with Notch signaling, and represses VEGF responsiveness, tip cell formation, and sprouting, rather than promoting sprouting of ECs [25]. Moreover, TGFβ produced by some tumors has been found to decrease endothelial VEGFR2 expression via ALK5 signaling [26].

### 1.1.2 Inhibitors of angiogenesis

There are also several proteins required for negatively regulating angiogenesis in order to prevent vascular chaos and tumor growth.

(1) Thrombospondin-1 (TSP-1)

TSP-1 is the most notable anti-angiogenic factor, which acts primarily by binding to its EC-expressed receptor CD36, resulting in the induction of EC death[27]. TSP-1 can also directly inhibit angiogenesis by interacting with other endothelial specific receptors (CD47, integrins, HSPG, and low-density lipoprotein receptor-related protein/LRP) to affect cell viability[28]. It has also shown that TSP-1 decreases the mobilization of circulating ECs and their progenitors to angiogenic sites [29]. Moreover, TSP-1 can abrogate the proliferative and chemotactic signals of both VEGF and bFGF [30, 31].

(2) Platelet factor-4 (PF4)

Platelet factor 4 (PF4) is a member of the C-X-C chemokine family, which is found in platelets and megakaryocytes [32]. Recent studies have contributed to a more clear definition of the role of PF4 in anti-angiogenic effects in vitro and in vivo. PF4 effectively inhibits the proliferation of vascular ECs in vitro by blocking cell cycle progression during S phase [33]. In addition, EC migration and tube formation in three-dimensional collagen type I gel are also blocked by PF4 [34]. In vivo studies have demonstrated that PF4 block angiogenesis in

matrigel plug implanted mice [35]. The main mechanism, through which PF4 executes the anti-angiogenic roles, has been clearly established, and is via the actions on FGF family memebers [34, 36]. Thus, PF4 binds directly to angiogenic growth factors and destabilize their three-dimensional structure. Furthermore, the intracelluar mechanism, by activating chemokine receptors [37], have also been identified.

(3) Angiostatin

Angiostatin is the first reported angiogensis inhibitor, which is not a novel protein molecule coded by novel DNA sequences. Instead, it is an internal proteolytic fragment of plasminogen. Angiostatin was first isolated from a subclone of Lewis lung carcinoma, and it inhibits primary and metastatic tumor growth by blocking tumor angiogenesis [38]. The fact that angiostatin has not been found in healthy tissues suggests that it remains a tumor-specific angiogenesis inhibitor. Angiostatin is an EC-specific inhibitor, which can interfere with an angiogenic process at various steps, including EC proliferation, migration [39], survival [40], and can block the angiogenic signaling pathway triggered by various angiogenic factors, including VEGF and FGF2 [41].

(4) Endostatin

Endostatin was isolation from a murine hemangioendothelioma by the same strategy that employed to find angiostatin in the same group, which is also the proteolytic fragment of collagen XVIII [42]. Endostatin is a well studied endogenous angiogenesis inhibitor that potently inhibits the growth of various tumors in mice. Endostatin mainly suppresses pathological angiogenesis , but not affect wound healing or reproduction [43].

Gene expression profile analysis shows that endostatin downregulates a number of angiogenic factors, including VEGF-A and FGF-2, and upregulates other known endogenous angiogenesis inhibitors such as maspin and TSP-1 [44]. Moreover, integrins αvβ5,αvβ3 and α5β1 have been linked to the antiangiogenic activities of endostatin [45]. Because some of these integrins are selectively expressed in growing ECs, it may explain the specific inhibitory activity of endostatin on angiogenic ECs.

### 1.1.3 Matrix metalloproteinase system in angiogenesis

The degradation of the basement membranes is an essential requirement for the formation of new vessels, and is mediated by matrix metalloproteinases (MMPs). MMPs are a family of enzymes that proteolytically degrade various components (such as collagens, laminins, elastin, and fibronectin) of the extracellular matrix (ECM) [46]. MMPs include collagenases (MMP-1, MMP-8 and MMP-13), stromelysins (MMP-3, MMP-10 and MMP-11), gelatinases (gelatinase A or MMP-2; gelatinase B or MMP-9), other MMPs and membrane-type MMPs (MT- MMPs) [47]. MMPs contribute in many ways to both pro- and antiangiogenic processes.

MT1-MMP was identified as the fibrinolysin responsible for degrading and remodeling the fibrin matrix [48]. MMP-1 is responsible for the initiation of degradation of interstitial collagen types I-III under physiological conditions, and vascular endothelial growth factor (VEGF) induces MMP-1 expression in human ECs [49]. MMP-7 (matrilysin) enhances EC proliferation, up-regulates endothelial expression of MMP-1 and MMP-2, and induces angiogenesis in vivo [50, 51]. Exogenous MMP-9(gelatinase B/type IV collagenase) has been shown to enhance EC growth in vitro [52]. However, MMP-7 and MMP-9 also revealed anti-angiogenic activities. It has been shown that MMP-7 and MMP-9 hydrolyze human plasminogen to generate angiostatin fragments [53], which are known to be a very potent angiogenesis inhibitor. MMP-12(a macrophage-derived metalloelastase) is also responsible for the generation of angiostatin in Lewis lung carcinoma [54].

MMPs' proteolytic activities are controlled by a family of proteins called tissue inhibitors of metalloproteinases (TIMPs). TIMP family regulates ECM turnover and tissue remodeling by forming tight-binding inhibitory complexes with the MMPs.

## 1.2 PLATELET PHYSIOLOGY

Platelets are anucleated cells shed from megakaryocytes in the bone marrow (Figure 1). They circulate in blood as small discoid cells, and are the smallest in size among the circulating blood cells, only 2.0 to 5.0 μm in diameter, 0.5 μm in thickness, and with a mean cell volume of 6 to 10 fl (femtoliters) [55]. The human body produces approximately 100 billion platelets per day. Platelets circulate at a cell concentration of around 200–300×$10^9$/L under physiological conditions, and their lifespan in the circulation is about 10 days in humans.

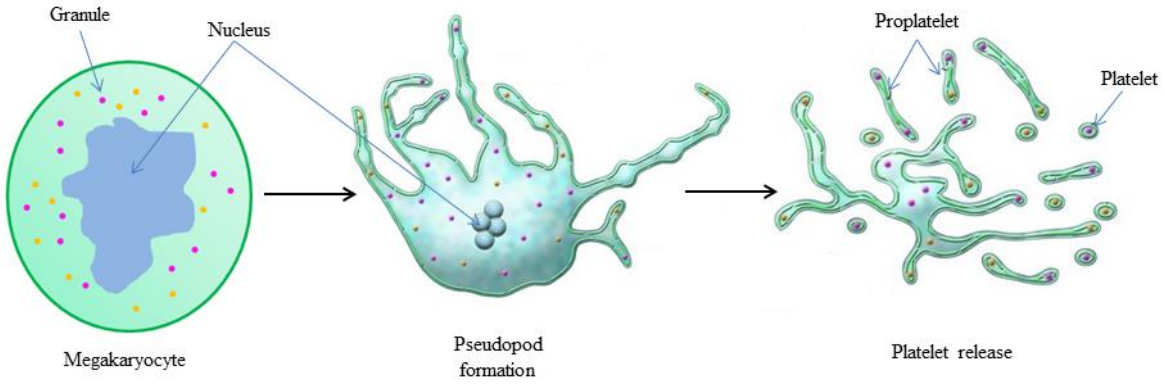


***Figure 1*: Schematic diagram of platelets production from megakaryocyte. (Modified from Sunita R et al., 2005)**

Platelets are usually in a quiescent state in circulation, and become activated by exposure to subendothelial ECM and/or soluble activators. They adhere to the damaged vessel wall, build up platelet aggregates, and form platelet thrombus. These processes restore integrity of the vessel wall and stop bleeding, but may also lead to occlusive thrombus formation under pathophysiological conditions [56]. Platelets function is highly associated with their structure/components.

### 1.2.1 Platelet membrane adhesive proteins

(1) Glycoprotein IIb/IIIa (GPIIb/IIIa)

GPIIb/IIIa, also known as αIIbβ3, is the major and only integrin expressed uniquely on platelets, and is essential for platelet aggregation [57]. The central function of αIIbβ3 is the capacity to undergo activation, which is a transition from a low-affinity state (resting state) to a high-affinity state (active state) for its extracellular ligands. αIIbβ3 binds to fibrinogen or multivalent von Willebrand factor (vWF) [58], which can bridge platelets together to form aggregates.

(2) Glycoprotein Ib-IX-V (GPIb-IX-V)

GPIb-IX-V complex is the second common receptor on platelets after integrin αIIbβ3, and is pivotal platelet receptor in initiating and propagating hemostasis and arterial thrombosis. The major function of GPIb-IX-V receptor is to mediate platelet adhesion to subendothelial matrix, ECs or leukocytes, and assembly of platelet procoagulant activity. Von Willebrand factor (vWF) is an important ligand of GPIb-IX-V, which mediates the first critical step in platelet adhesion. Thrombospondin (TSP) is another identified ligand for GPIb-IX-V, and the interaction is capable of supporting platelet adhesion at high shear rates in the absence of VWF [59]. In addition to the major collagen receptors GPVI and the integrin α2β1, GPIb-IX-V is also an indirect collagen receptor on platelets through VWF bridging interactions [60]. GPIb-IX-V has also been suggested as a counter receptor for P-selectin [61].By binding coagulant factors, such as α-thrombin/factor IIa, factor XI, factor XII, factor VIIa, GPIb-IX-V receptors also contribute importantly to platelet procoagulant activities.

(3) Glycoprotein VI(GPVI)

GPVI, a member of the immunoglobulin superfamily, is exclusively expressed on platelets, and is an other major established platelet receptor for collagen,in addition to α2β1 [62]. FcR γ-chain has been recognized as a critical binding and signaling partner of GPVI [63]. The initial event in signaling by GPVI is phosphorylation of a conserved motif in the FcR γ-chain known as an ITAM. GPVI is partially or completely excluded from lipid rafts in resting platelets but translocated to these domains upon ligand engagement [64]. Platelet adhesion to collagen requires platelet activation though GPVI, subsequent inside-out regulation of activation of integrin α2β1, and is reinforced by released second-wave mediators ADP and thromboxane $A_2$ ($TXA_2$) [60].

(4) α2β1

α2β1, also known as GPIa/IIa on platelets, is the second most important platelet integrin after αIIbβ3, and is a major collagen adhesion receptor. The patients with myeloproliferative disorder have been shown to have a deficiency in platelet α2β1, and lack aggregation and adhesion responses to collagen [65]. Antibodies to α2β1 can prevent the aggregation response when platelets are activated by collagen in a stirred suspension, despite the presence of GPVI on the platelets [66].

(5) P-selectin(CD62P)

P-selectin is an important adhesion receptor present on platelets, and is stored as an integral protein of granule membrane in platelets and Weibel-Palade bodies in ECs [67]. Upon cellular activation, fusion of the granules with the external membrane exposes P-selectin on the cell surface. P-selection mediates rolling of platelets [68], the interaction between platelets and leukocytes, and between platelets and ECs via PSGL-1 [69-71]. Furthermore, P-selectin stabilizes initial platelet aggregates formed by GP IIb/IIIa-fibrinogen interactions, allowing the formation of large platelet aggregates [72].

(6) α6β1

α6β1 is the main laminin-binding integrin on platelets, despite a number of receptors with the potential to bind laminins express on platelets, such as integrins αvβ3, α2β1 and GPVI [73]. Upon laminin binding, α6β1 activates platelets by initiating signals promoting cytoskeletal reorganization and filopodia emission [73]. Evidence obtained from α6β1 knockout mice has confirmed that the adhesion of platelets to laminins is critically dependent on integrin α6β1, not to VWF, fibrillar type-I collagen, fibrinogen, or fibronectin [74].

(7) CD40 and CD40L

CD40 is a membrane glycoprotein belonging to the tumor necrosis factor (TNF) receptor superfamily. It is expressed on B lymphocytes, monocytes, dendritic cells, ECs, and also constitutively expressed on platelets [75]. The ligand for CD40 — CD40L (CD154) is also a glycoprotein from the TNF family. Platelets express membrane-bound CD40L [76], and the surface-expressed CD40L is cleaved from platelets, which subsequently generates a soluble fragment (sCD 40L) in response to activation. CD40L/sCD 40L can be recognized by CD40 expressed on other cells, even by platelets themselves.

CD40L-CD40 interaction has been recognized to be of critical importance in adaptive immune response, inflammation, atherosclerosis, and thrombosis. Furthermore, CD40 ligation has also been demonstrated a role in platelet activation, as evidenced by the enhanced CD62P expression and release of dense and α-granules after incubated with trimeric form of sCD40L (sCD40LT) [75].

### 1.2.2 Platelet membrane receptors

(1) Thrombin receptors—PAR1 and PAR4

The serine protease thrombin is the most potent activator of human platelets and is critical for maintaining normal hemostasis. Thrombin receptors, also as known protease-activated receptors (PARs), have a distinctive mechanism of activation, involving specific cleavage of the N-terminal extracellular domain of the receptor, which exposes a new N-terminus that, by refolding, acts as ligand to the receptor [77].

Four PAR receptors, termed as PAR1-4, have been characterized. Among them, PAR1 is the major thrombin receptor on human platelets. PAR3 is expressed instead of PAR1 on mouse platelets but is not present in humans. PAR4 is a lower-affinity thrombin receptor on platelets that can form a heterodimer with the high- affinity PAR1 receptor [78]. PAR2, a trypsin/tryptase/ matriptase receptor,is not expressed on platelets. The PAR-specific peptide agonists and antagonists have been identified [79], such as TFLLRN and SFLLRN for PAR1, AYPGKF for PAR4.

(2) ADP receptors—P2Y12 and P2Y1

ADP is a weak platelet agonist. However, ADP secretion from platelet dense granules is a critical autocrine and a positive feedback mechanism in the amplification phase of platelet activation. Eight ADP receptors, also known as P2Y receptors, have been identified: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. Human platelets express at least two ADP receptors, P2Y1 and P2Y12. P2Y12 expression is far more than P2Y1 [80]. The Gq-coupled P2Y1 receptor mediates a transient rise in cytoplasmic $Ca^{2+}$, platelet shape change, and rapid reversible aggregation. The Gi-coupled P2Y12 receptor exerts its effect via inhibiting adenylyl cyclase, leading to decreased intracellular cAMP levels. The latter enhances platelet activation, and amplifies the platelet aggregation response [81].

(3) Thromboxane $A_2$ ($TXA_2$) receptor: TPα and TPβ

$TXA_2$ is generated from its precursor arachidonic acid through the cyclooxygenase pathway during platelet activation. Human TP receptors exist in two isoforms, TPα and TPβ, which are encoded by the same gene, and are the results from alternative splicing. Once synthesized, $TXA_2$ can diffuse outside and activate other platelets, amplifying the initial platelet activation. TP deficient mice have been shown to be associated with a prolonged bleeding time, and their platelets were unable to aggregate in response to $TXA_2$ [82]. Aspirin can abolish $TXA_2$ generation, and impairs platelet responses to thrombin and ADP blocking secondary $TXA_2$ generation.

(4) Serotonin receptor: 5-HT2A

Serotonin (5-hydroxytryptamine, 5-HT) is a major component of dense granules and is released upon platelet activation. The serotonin 2A receptor (5-HT2A) is the major platelet serotonin receptors, which are coupled to G proteins, and its occupation by serotonin leads to calcium signaling. 5-HT2A activates PLC through Gq and leads to an accumulation of IP3, and the increase in cytoplasmic IP3 causes a release of calcium from intracellular endoplasmic reticulum stores [83]. 5-HT2A has been implicated in mental disorders with complex etiologies that are still not clearly understood. The results from Hrdina and the colleagues [84]suggested that upregulation of platelet 5-HT2 receptors in major depression, particularly in females. However, the study from another group showed that there were no alternations of the functional status of platelet 5-HT2A in major depression [85].

(5) Tetraspanins

Tetraspanins are a group of glycoproteins that have important functions in signal transduction across the cell membrane in complexes with other membrane receptors. By organizing various functional molecules, tetraspanins are implicated in multiple biological processes, including cell fusion, migration,proliferation and differentiation. Platelets contain several members of tetraspanins, including CD9 (Tspan29), CD63 (Tspan30), CD151 (Tspan24, PETA-3), and CD82[86].

CD9 is the main member of the tetraspanin group present on platelets, and is densely expressed on platelets. C. BRISSON et al.[87] have provided evidence showing that, at the ultrastructural level, CD9 and GPIIb-IIIa are co-localized on activated platelet pseudopods and α-granule membranes, using electron microscopy and different immunolabelling procedures. Another platelet-expressed tetraspanin CD151 has been suggested for the interaction with α6β1[88]. CD151 is also the positive regulator of αIIbβ3 signaling, Lau LM et.al [89] showed that CD151-deficient mice re-bled in tail bleed assays, and that the mice were found to be accompanied by a dramatic defect in platelet spreading on fibrinogen, as well as a coimmunoprecipitation was observed between CD151 and αIIbβ3.

### 1.2.3 Platelet secretory granules

Platelets contain three major types of secretory organelles, i.e., α granules, dense granules (also referred as δ granules or dense bodies) and lysosomes.

(1) α-granules

The α-granules are unique to platelets, and are the most abundant platelet granules. There are approximately 50-80 α-granules per platelet, with the size ranging from 200 to 500 nm. The α-granules contain a variety of high-molecular-weight proteins, such as adhesive proteins (vWF, fibrinogen), coagulation factors (FactorsV,XI, XIII), and many angiogenesis-regulating factors (such as VEGF, PDGF, SDF-1α, and PAI-1).

(2) Dense granules

The dense granules contain small molecules, such as calcium and adenine nucleotides (ADP and ATP). The role of dense granules in thrombus formation has been inferred by using knock-out mice, in which thrombus formation in dense granule deficient mice is dramatically inhibited [90].

(3) Lysosomes

The lysosomes contain many acid hydrolases and cathepsins as cargo, and express CD63 and LAMP-2 on their membrane. Platelet lysosome function is not well studied. They may serve a role in endosomal digestion, as observed in nucleated cells.

### 1.2.4 Platelet activation

When platelets are circulating through vessels lined with an intact, healthy endothelium, platelets remain their resting state. The absence of activating factors and the release of prostacyclin (prostaglandin $I_2$) by the healthy endothelium maintain a quiescent state of platelets. However, when blood vessels are injured, platelets can be activated by exposure to a wide range of stimuli, such as collagen, $TXA_2$, ADP, and thrombin.

Upon activation, platelets undergo a series of morphological and biochemical changes: 1) Shape change. When platelets are activated, resting platelets change their discoid shape into to a more amorphous form with projecting pseudopodia, increasing their surface area available for adhesion to ECM and to other cells, including platelets themselves. 2) Adhesion and aggregation. Activated platelets bind to VWF, mainly via their VWF receptors GPIb/V/IX and GPIIb/IIIa, and to collagen via corresponding platelet receptors GPVI and α2β1, leading to platelet adhesion on the injured vessel wall. The binding of fibrinogen by activated GPIIb/IIIa (αIIbβ3) causes platelets to adhere to each other. Hence, platelets adhere to one another and to subendothelial matrix exposed after vessel injuries, forming the initial platelet aggregates or platelet plug. 3) Secretion. During platelet activation, platelets secrete a large amount of bioactive molecules from dense and α-granules, which can then act on other cells and on adjacent platelets, as well as on activated platelets themselves in an autocrine manner. The latter constitutes a positive feedback cascade to amplify platelet activation. 4) Coagulation reactions. Intense platelet activation results in the exposure of phosphotidylserine (PS), which provides attachment sites for the coagulation factors FVa and FXa to form prothrombinase complex. In turn, the product of coagulation cascade thrombin reinforces platelet activation, and contributes to the stabilization of the thrombus.

### 1.2.5 Platelet proteome and microRNAs

Despite lacking genomic DNA, and thereby being incapable of transcription of nuclear materials, platelets inherit considerable amount of RNAs from their precursor megakaryocytes. Andrew. Weyrich's group found 9538 RefSeq transcripts (at a 0.3 RPKM cut-off) in human platelets, by using next-generation RNA sequencing (RNA-seq) [91]. Moreover, platelets contain rough endoplasmic reticulum and ribosomes [92]. Therefore, platelets are equipped with a full functional machinery of protein synthesis. Indeed, emerging evidence indicates that megakaryocytes invest platelets with thousands of messenger RNAs (mRNAs) [93]. Hence, they can use mRNAs as templates for de novo protein syntheses in response to stimuli [94], suggesting that the platelet proteome is much more dynamic than previously believed.

Human platelets contain an abundant and diverse array of microRNAs, as well as the main cytoplasmic protein components of the microRNA pathway, including Dicer, TARBP2 (TAR RNA-binding protein 2) and Argonaute 2(Ago2). Pre-microRNAs are also detected in human platelets. Patricia Landry et.al first revealed that platelets contain the Dicer•TRBP2 complex, which functions in processing exogenous microRNA (miRNA) precursors to form mature miRNAs. Furthermore, they found that microRNP (microRNA-protein complex) effector complexes, formed by Ago2 and its associated microRNAs, exist in platelets, and can effectively control specific transcripts [95].

Increasing evidence indicates that the function of platelets is not just mediated by or involves a repertoire of proteins inherited from their megakaryocytic precursor cells, but also involves in sequence-specific, post-transcriptional regulatory mechanisms of gene expression.

## 1.3 THE ROLE OF PLATELETS IN ANGIOGENESIS

Among the earliest evidence showing that platelets are crucial for human hemostasis and thrombosis, more recent evidence has highlighted an important role of platelets in angiogenesis. Saba SR et al showed that platelets promoted the growth of ECs [96]. Platelets can promote rat aortic angiogenesis [97] and tube formation of ECs on matrigel [98]. The effect of platelets on would healing [99] and tissue repair[100] have also been documented since 1990s. Moreover, it has been shown that platelets enhanced homing of EPCs to subendothelial matrix [101] and induced development of endothelial progenitor cells into mature ECs [102].

Until the work by Denisa Wagner's lab, the role of platelets in angiogenesis in vivo was largely uncharacterized, despite earlier studies showed that platelets stimulated angiogenesis in vitro. Denisa Wagner and colleagues' paper in PNAS 2006 showed that platelet-enhanced angiogenesis using two different in vivo assays (the cornea micropocket assay and the Matrigel model) in four animal models [103]. In their study, the thrombocytopenic mouse model showed that the absence of platelets led to a decreased number of new vessel formation in vivo. Moreover, platelet adhesion deficiency also led to abnormal angiogenesis using a GPIb/IL4R transgenic mouse model. Their results suggested that platelets and their adhesive function support angiogenesis in vivo.

After proving that platelets can support angiogenesis in vivo, Denisa Wagner's group further showed that platelets can maintain vessel integrity in tumors [104]. In that study, they induced severe acute thrombocytopenia in mice bearing subcutaneous Lewis lung carcinoma or B16F10 melanoma. They found that thrombocytopenia led to bleeding in tumors, and that platelets continuously support tumor vascular homeostasis by regulating the stability of tumor vessels through the secretion of their granule content.

Although a number of earlier studies have shown strong evidence of angiogenic functions of platelets, the full understanding of the mechanisms remains obscure. The current understanding of platelet angiogenic activities mainly focus on the following aspects:

1. Platelet-released angiogenic factors

As a rich source of angiogenic regulators, platelets α-granules harbor a panel of angiogenic regulators, such as VEGF (vascular endothelial growth factor), PDGF (platelet derived growth factor), FGF (fibroblast growth factor), TSP-1 (Thrombospondin-1) and PF4 (platelet factor 4)/CXCL4, among others. Platelets can rapidly respond after interaction with stimuli and release cargos from their storage granules.

Some of these cargos have opposing activities. For instance, VEGF has been well recognized as the most important and potent pro-angiogenic regulator, whilst TSP-1 is a well-established negative regulator of angiogenesis. It is very interesting to investigate how cargoes with counteracting functions efficiently elicit physiological responses. There are three main hypothesis concerning this issue: A) α-granule contents are released indiscriminately, and the physiological activity of platelet releasate is dictated solely by stoichiometry and kinetics of their activities on target receptors; B) specific platelet cargoes are stored in different subsets of α-granules that are released only in response to particular agonists; C) cargos may be randomly distributed among granules but segregated within the granule and released preferentially in response to different agonists or in an activation intensity-dependent manner.

Ma et al.demonstrated that PAR1 activation led to release of VEGF, and that, in contrast, PAR4 activation stimulated endostatin release [105]. Italiano's lab has confirmed those findings, and proposed that platelets store pro-angiogenic regulators and anti-angiogenic regulators in separate α-granules [106]. Recent work from our laboratory also supports this concept. However, based on 3-dimensional (3D) confocal fluorescence microscopy, Kamykowski et al.failed to show evidence of co-clustering of angiogenic regulators into functionally distinct α-granule populations using a super-resolution analysis of 15 different human α-granule proteins and quantified 28 different pair-wise comparisons [107]. Moreover, there is a recent report showing that PAR1 and PAR4 stimulation induced similar release of platelet angiogenic regulators [108].

2. Platelet microRNAs and their regulation of protein synthesis

MicroRNAs (miRNAs) are endogenously expressed short noncoding RNAs (21–24 nucleotides), which are involved in posttranscriptional regulation of cellular messenger RNA (mRNA). Platelets are devoid of a nucleus and lack genomic DNA. Although being incapable of transcribing nuclear genes into mRNAs, platelets inherit proteins and mRNAs from their precursor cells (megakaryocytes). Because platelets contain rough endoplasmic reticulum and ribosomes, platelets can therefore use mRNAs as templates for de novo protein synthesis. Patricia Landry, et al demonstrated that platelets harbor a gene-regulatory pathway based on miRNAs [95]. By using in vitro RISC (RNA-induced silencing complex) activity assays, they showed that platelet miRNAs can mediate RNA silencing, and that Ago2 protein is the endogenous miRNA effector complex in platelets. This is the first publication that proved the existence of functional miRNA pathway components in human platelets. This group mainly focuses on microRNA-223 (the most abundant miRNAs in platelets) associated-Ago2

complexes, which may regulate the P2Y12 expression [95]. It should be noted that platelets can initiate de novo protein synthesis of plasminogen activator inhibitor 1 (PAI-1) [109], thrombospondin-1 (TSP-1) [94], and stromal cell-derived factor-1 (SDF-1) [110] upon stimulation. Therefore, it would be very interesting to investigate if de novo synthesis of these angiogenic factors can be regulated by platelet microRNAs.

3. Angiogenic involvement of platelet derived microparticles

Many cells, such as endothelial cells, leukocytes, erythrocytes, and platelets, can shed the small plasma membrane vesicles/microparticles (0.05-1 μm). Platelet-derived microparticles (PDMP), shed from platelets or derived directly from their progenitor cells---megakaryocytes, constitute the major pool of microparticles (labeled with anti-CD41 or anti-CD42b) circulating in the blood.

The best characterized function of PDMP s is their participation in blood coagulation, by providing a source of tissue factor and negatively charged surface where coagulation factor complexes assemble. Furthermore, some studies have demonstrated that platelet-derived microparticles can function as a transporter and delivery system for bioactive molecules. It has been shown that PDMPs affect target cells by stimulating them via surface expressed ligands, by transferring surface receptors to other cells, or by delivering cytoplasmic proteins and RNAs (including microRNAs) to recipient cells. Kim et al have demonstrated that PDMPs promote the proliferation and survival, migration, and tube formation of human umbilical vein endothelial cells (HUVEC) [111]. PMPs exert the effect mainly through their lipid components, because treating PDMP with activated charcoal (known to remove the lipid impurities) can significantly reduce angiogenic activity of PDMPs [111]. Brill et.al have demonstrated that PDMPs induce sprouting both in vitro (using rat aortic ring model and cell invasion test) and in vivo (using agarose bead transplantation) to a degree comparable with that of whole platelets [112]. The same study has identified VEGF, bFGF, and PDGF as the mediators for this effect. Prokopi M et.al showed that depletion of PDMPs from the conditioned medium of EPC cultures attenuated endothelial tube formation in the Matrigel assay, and that integrin αIIbβ3 seems to be involved in this proangiogenic effect of PDMPs [113].

4. Roles of platelet membrane components in angiogenesis

Platelet membrane components seem to contribute to endothelial angiogenic activities. The first evidence of platelet-regulated angiogenesis through membrane component interactions was from the observation that physical presence of platelets but not the platelet releasate is necessary for platelet-promoted ECs tube formation in Matrigel [98]. It has also been shown that platelets promote EC proliferation through interactions via both P-selectin and CD40L [114].

Platelets rapidly adhere to inflamed ECs and/or exposed subendothelial extracellular matrix. The adhered platelets have been show to enhance the recruitment of circulating EPCs at the

sites of vessel injury [115] . With the close contact of platelets with ECs/EPCs, it would be of great interest to investigate how direct cell-cell contact between platelets and ECs/EPCs influence the angiogenic activities of ECs and EPCs, and how these interactions influence the remodeling process of thrombus.

# 2 AIM OF THIS STUDY

Overall aim of the thesis work is to investigate the regulatory functions of platelets in angiogenesis. Specifically, we aimed to:

1) Study if and how selective platelet release of pro- and anti-angiogenict factors affect angiogenic activities of EPCs.

2) Elucidate how platelet membrane components regulate angiogenesis.

3) Investigate if and how platelet miRNAs regulate the protein synthesis of angiogenic regulators in platelets and platelet-dependent angiogenesis.

4) Demonstrate if and how platelet dysfunction of diabetes mellitus may influence platelet angiogenic activities in diabetic patients.

# 3 METHODOLOGY

## 3.1 PLATELETS AND CELL PREPARATION

### 3.1.1 Platelet isolation, activation and releasate preparation

(1) Platelet isolation

To prevent the activation of platelets during sample processing, blood samples were handled with care (e.g., avoiding fast pipetting or vigorous shaking). In addition, the platelet inhibitors were used.

a. Blood fractionation

The first step of this procedure was obtaining platelet-rich plasma (PRP) by centrifugation (190 ×g for 20 min). After the first spin, three distinct layers can be observed:

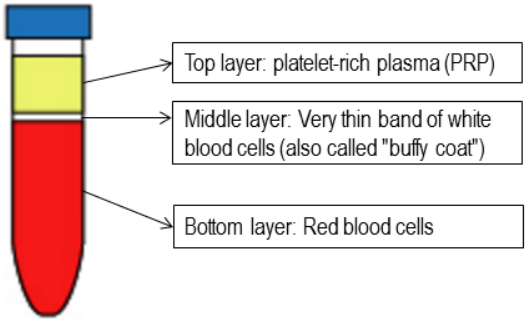


***Figure 2*. Illustration of blood fractionation after centrifugation.**

b. Washed platelet preparation

♠: Transfer upper two-thirds of the PRP into a new plastic tube without disturbing the buffy coat layer, to avoid contamination of other blood cells. Prostaglandin $E_1$ ($PGE_1$, 1 μM) or prostaglandin $I_2$ ($PGI_2$, 1μM) was added to minize platelet activation.

♠: Mix very gently by inverting the tube slowly, which is followed by spinning at 190 ×g for 20 min to pellet contaminating red and white blood cells.

♠: Transfer the supernatant into a new plastic tube, and then pellet platelets by high speed centrifugation at 900 ×g for 10 min. Discard the supernatant, and then carefully and slowly re-suspend the pellet to a desired concentration.

In some experiments, platelets were purified by leukocyte depletion using CD45 Dyna beads before pellet.

Platelets from DM patients are hyperactive, and have a tendency to clot during isolation processes. Hence, we have modified the above protocol by lowering centrifugation speed and adding Acid-Citrate-Dextrose (ACD) buffer (10%, v/v) plus $PGI_2$ before pelleting.

(2) Platelet activation

After the recovery of platelets reactivity (30 min, 22°C), whole blood or washed platelet samples were stimulated with vehicle or an indicated agonist (PAR1-AP, PAR4-AP, or thrombin) with optimal concentration for certain time at 37°C. The activation rate was determined by flow cytometry.

(3) Preparation of platelet releasates

Washed platelet activation was terminated by an ice bath, and the samples were then centrifuged at 1000 ×g for 10 min at 4°C. The supernatants were collected and centrifuged again at 15000 ×g for 10 min at 4°C. Afterwards, the releasates were aliquoted and stored at -80°C for further use.

### 3.1.2 Endothelial progenitor cell (EPC) generation and culture

EPCs, also referred as endothelial colony forming cells (ECFCs) or late outgrowth endothelial progenitor cells, are blood culture-derived cells that can develop into mature endothelial cells and are capable of de novo vessel formation. They share many similar properties with endothelial cells (ECs), and can serve as an autologous source for vascular regeneration and endothelial reparation. Hence, the present thesis work used EPCs to investigate platelet angiogenic activities.

Venous blood was laid onto the top of the Histopaque 1077 (1:1, v/v; Sigma-Aldrich) gently, and centrifuge at 500 ×g for 30 minutes at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected from the middle layer, and washed twice with PBS. The PBMCs were resuspended with EGM-2 complete medium (EBM-2 basal medium supplemented with 10% FBS and a growth factors cocktail). Then, the PBMCs were seeded at a density 2–4×$10^6$ cells per well of a fibronectin-coated 24-well culture plate, and cultured in a humidified incubator under 5% $CO_2$ at 37°C. The nonadherent cells were removed after 4 days culture by replacing the medium, and the remaining cells were continually cultured. Medium was changed every 3 days until the first passage.

### 3.1.3 MEG-01 cell culture and transfection

Platelets are known as a difficult cell type for transfection. The human megakaryoblastic leukaemia cell line MEG-01 cells display phenotypic properties that closely resemble those of megakaryoblasts, but not other blood cell lineages. Therefore, we selected MEG-01 cells to investigate the regulatory effects of platelet miRNAs on target protein synthesis.

(1) MEG-01 cell culture

MEG-01 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified incubator under 5% $CO_2$ at 37°C. Culture medium was replaced every 2 or 3 days, and the cells were subculture for 2 or 3 days, with a ratio at 1-1.7 ×$10^5$ cells/ml before transfection.

(2) MEG-01 cell transfection

MEG-01 cells ($1\times10^6$) were transfected with 100 nM of miR-27b-3p mimics, as well as the corresponding negative control using Amaxa® Cell Line Nucleofector® Kit C and Nucleofector® Device as per manufacturer's instructions. Cells were incubated for 72 hours after transfection prior to western blotting analysis. Transfection efficiency was estimated in parallel by co-transfection of a GFP-expressing plasmid and flow cytometric analysis of GFP-expression 24 hours post Nucleofection.

## 3.2 IN VITRO CELL FUNCTIONAL ASSAY

### 3.2.1 Cell proliferation assay based on cell counting kit (CCK)-8

CCK-8 assay is a sensitive colorimetric assay for the determination of the number of viable cells during cell proliferation. Briefly, the detached cells were resuspended in the complete medium, and cultured in triplicates in a 96-well flat-bottom plate with or without treatment. After 18- and 48-h incubation, the CCK-8 colorimetric reagent WST-8 was added to each well and further incubated for 3 h. Then, the absorbance was measured at 450 nm using a microplate reader.

### 3.2.2 Apoptosis and cell cycle analyses

Annexin V staining, paired with propidium iodide (PI) is widely used to identify apoptotic stages of cells by flow cytometry. Viable cells are both Annexin V and PI negative. Cells in the early stage of apoptosis are Annexin V positive but PI negative, while cells in late apoptosis or necrotic cells are both Annexin V and PI positive.

### 3.2.3 Cell migration assay

The EPC migratory function was evaluated using a modified Boyden chamber assay, which is a useful tool to study cell migration and cell invasion. The Boyden chamber consists of a cylindrical cell culture insert nested inside the well of a cell culture plate. The insert contains a polycarbonate membrane at the bottom with a defined pore size. Cells are seeded in the top of the insert in serum-free media, while serum or similar chemoattractants are placed in the well below. Migratory cells move through the pores toward the chemoattractant below. Cells remaining on the upper surface of the transwell membrane were wiped away gently with a cotton ball, and the cells that had migrated to the lower surface were fixed and stained with Giemsa. The magnitude of EPC migration was evaluated by counting the migrated cells in 10 random high-power microscope fields.

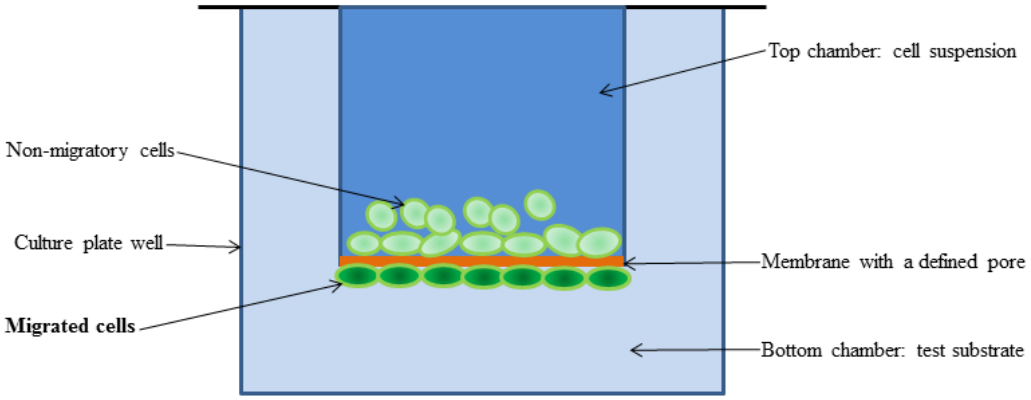


*Figure 3.* **An illustration of cell migration assay using a Boyden chamber.**

### 3.2.4 In vitro tube formation on Matrigel

The tube formation assay can be used to identify genes and pathways that are involved in the promotion or inhibition of angiogenesis in a rapid, reproducible, and quantitative manner. Endothelial or endothelial progenitor cells are mixed with conditioned media and plated on Matrigel matrix (basement membrane extract). The capillary like structures occurs within hours in response to angiogenic signals found in conditioned media, and tubes can be visualized using a phase contrast inverted microscope, or the cells can be treated with calcein AM prior to the assay and tubes visualized through fluorescence or confocal microscopy. The number of branch sites/nodes, loops/meshes, or number or length of tubes formed can be easily quantified as a measure of in vitro angiogenesis.

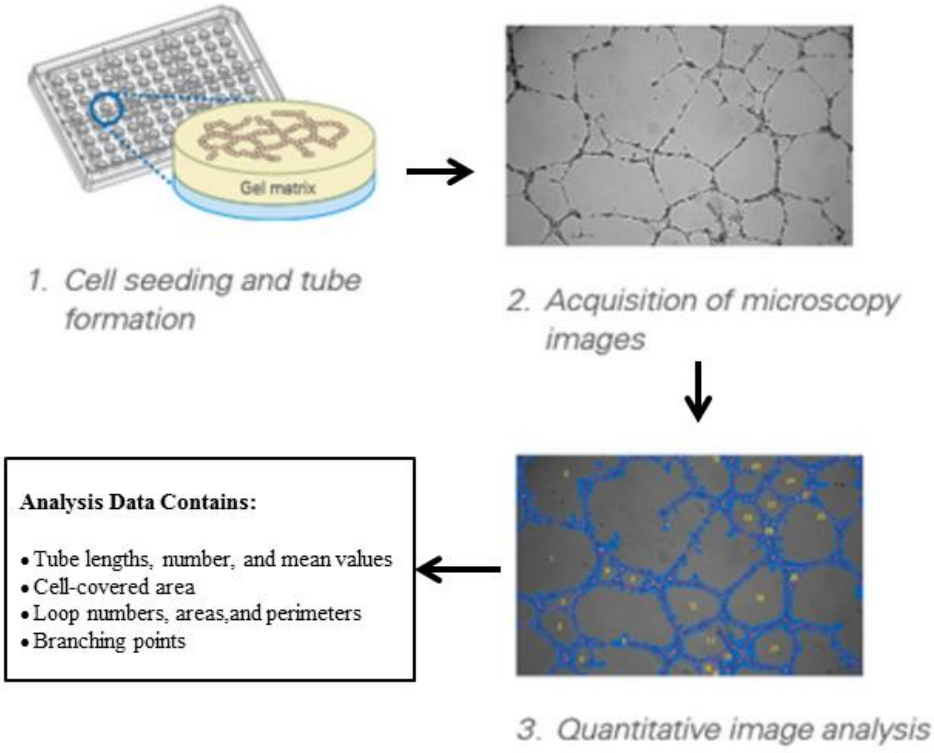


*Figure 4.* **Tube formation assay on matrigel.** (Modified from ibidi.com)

## 3.3 MOLECULAR ASSAY

### 3.3.1 Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical and immunofluorescent characteristics of single particles, usually cells, as they flow in a fluid stream through a laser beam. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the individual antigenic markers of the cell. In a mixed population of cells, different fluorochrome-conjugated antibodies can be used to distinguish different subpopulations. The antigenic marker staining combined with FSC and SSC data can be used to identify which cells are present in a sample and to count their relative percentages. In our study, we use flow cytometry to identify the characterization of EPCs, platelet activation and purity, as well as GFP transfection efficiency.

### 3.3.2 RNA immunoprecipitation (RIP)

RIP is an antibody-based technique used to map in vivo RNA-protein interactions. The RNA binding protein (RBP) of interest is immunoprecipitated together with its associated RNA for identification of bound transcripts (mRNAs or non-coding RNAs). Transcripts are detected by real-time PCR, microarrays or sequencing. In the present study, Ago2 and its associated miRNA is our interest. Ago2 protein is a component of the RISC and mediates small interfering RNA (siRNA)-directed mRNA cleavage and microRNA translational suppression.

### 3.3.3 Microarray-based miRNA profiling

MicroRNA microarray technology is a powerful tool for high-throughput genome-wide miRNA expression profiling, and is widely used for screening for candidates. The basic principle is that the fluorescence-labeled RNA target hybridized with complementary oligonucleotide probes, which have been assembled on the chip surface. The signal is detected by laser scanning and quantified by fluorescence intensities that represent the abundance of miRNAs. In the present study, miRNA expression profiling was performed using Human Agilent's miRNA Microarray. Briefly, total RNA (100ng) were fluorescence-labeled with Cyanine 3-pCp, and hybridized with the arrays for 20 hours at 55 °C. Slides were scanned in an Agilent microarray scanner G2565BA and obtained images processed with Feature Extraction Software 9.5.3.1.

### 3.3.4 qRT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a technique that provides reliable detection and measurement of cDNA in a given sample based on the PCR. There are two common qRT-PCR methods: SYBR Green-Based qRT-PCR and TaqMan assay. The SYBR Green method detects the fluorescence signal from the intercalation of the SYBR Green dye with any double-stranded DNA (dsDNA), and this method has the advantage of only needing a pair of primers to carry out the amplification. TaqMan assay

uses a fluorogenic probe specific to target gene to detect target as it accumulates during PCR. A fluorescent reporter dye and a quencher are attached to the 5' and 3' ends of the TaqMan probe, respectively. The reporter dye emission is quenched when the probe is intact, and the DNA polymerase cleaves the reporter dye from the probe during each extension cycle. Once separated from the quencher, the reporter dye emits its characteristic fluorescence, thus enables the detection of fluorescence signal. For both methods, the amount of fluorophore released from the SYBR Green intercalation or the TaqMan probe is directly proportional to the amount of DNA template present in the PCR, thus provides a quantitative analysis of the gene or transcript.

In the present thesis work, the quantitation of mRNAs and miRNAs were performed using theSYBR Green and Taqman assay, respectively.

### 3.3.5 Western Blot

Western blot is a protein detection method based on antibody-antigen specific interaction. In the present work, the lysate or a given sample was separated in NuPAGE™ Novex™ 4-12% Bis-Tris protein gels and transferred to nitrocellulose membranes. The membranes were then incubated with antibodies against the target proteins after blocking non-specific bindings. Horseradish peroxidase (HRP) conjugated secondary anti-mouse or goat IgG were used to detect the target proteins by developing with HRP chemiluminescent substrate reagents. In the present studies, we applied western blot to detect the target proteins including Ago2 and TSP1, and β-actin or GAPDH was used as a loading control. Signals were visualized on Fujifilm LAS 4000 and protein expressions were quantified on the immunoblots using ImageJ.

### 3.3.6 Immunoassays (ELISA)

ELISA is a method that uses antigen-antibody reaction and color change to detect and quantify substances in biological fluids. Colorimetric sandwich ELISAs were performed in our studies, which quantify antigens between two layers of antibodies (capture and detection antibody). In simple terms, a capture antibody is pre-coated in a 96-well microplate, the target antigen is captured by this antibody upon incubation with experimental samples. A conjugated detection antibody that binds to a different epitope on the target analyte is used to complete the sandwich. A substrate solution is subsequently added to produce a signal that is proportional to the amount of analyte bound.

## 3.4 IN VIVO VASCULOGENESIS

Although in vitro angiogenesis assays (such as tube formation, migration and invasion assays) have the advantages of simplicity and repeatability, they lack the biologic complexity in vivo. Therefore, in vivo studies are more informative and more helpful for a thorough understanding of the complex process. In the present work, a mouse model of Matrigel implantation was used, as it is easy to perform and reproducible, and the progression of angiogenesis can be observed within a few weeks. In the assay, Matrigel was injected

subcutaneously, endothelial cells migrated into the Matrigel plug and form vessels. The angiogenic process can be accelerated or inhibited by adding different testing materials, to the Matrigel.

# 4 REUSLTS AND DISCUSSION

## 4.1 PAR1-STIMULATED PLATELET RELEASATE (PAR1-PR) MORE PROFOUNDLY PROMOTES ANGIOGENIC ACTIVITIES OF ENDOTHELIAL PROGENITOR CELLS THAN PAR4-PR

### 4.1.1 Both PAR1-PR and PAR4-PR had little influence on EPC proliferation

To study the influence of platelet releasates on EPC proliferation, EPCs were treated with three different concentrations (2.5%, 5% and 10%) of platelet releasate during 18 or 48 h cell culture. EPC proliferation was monitored using the colorimetric assay CCK-8, and cell cycle and apoptosis analysis of the cultured EPCs were also performed using Annexin V and PI staining.

The releasates were found to have no effect on EPC proliferation, as the presence of either PAR1-PR or PAR4-PR did not alter the absorbance readings of the CCK-8 colorimetric reagent WST-8 of the cultured cells as compared to the controls. Similarly, neither PAR1-PR nor PAR4-PR significantly influenced EPC cell cycle distribution in the G0/G1, S, or G2/M phase. Moreover, addition of either PAR1-PR or PAR4-PR to cultured EPCs did not alter the percentages of the total Annexin $V^+$ EPCs, which included early and late apoptotic cells, as well as necrotic cells. The latter indicates that the platelet releasates had no effects on apoptosis of cultured EPCs.

Our results showed that platelet releasates did not enhance EPC proliferation. The mechanisms behind the phenomenon may be complex, but the phenomenon likely represents a balance between pro- and anti-proliferative factors in platelet releasates. While platelet-released VEGF, bFGF, and PDGF can definitely enhance cell proliferation of EPCs, platelet releasates also contain high levels of PF4, TGFβ, and TSP-1, which are known to inhibit endothelial proliferation.

### 4.1.2 Both platelet releasates facilitated EPC migration

We performed a Boyden chamber chemotactic migration assay to study the effect of platelet releasates on EPC migration. The numbers of migrated EPCs were significantly increased in the wells containing PAR1-PR (relative increase by 25%) and PAR4-PR (29%) after 6h culture, and the numbers were further increase as the incubation time increased to 24 h. Moreover, there was no difference between the treatments with PAR1-PR and PAR4-PR on either observation time points.

### 4.1.3 PAR1-PR more profoundly increased EPC angiogenesis than PAR4-PR

We examined the effects of platelet releasates on angiogenic activities of EPC both in vitro and in vivo. The in vitro Matrigel tube formation model showed that both PAR1-PR and PAR4-PR mildly enhanced tube formation of EPCs in the complete medium containing 10%

FBS and a cocktail of growth factors, and that the enhancements of the two releasates were similar. Figure 4 shows that, when the basal culture medium (supplemented with only 0.5% FBS) was used, both PAR1-PR and PAR4-PR enhanced EPC tube formation more markedly, and notably, PAR1-PR more profoundly increased EPC tube formation than PAR4-PR (figure 5). These results suggest that the impacts of platelet releasates and the difference between the two platelet releasates are elicited more easily in the basal culture medium, which had a minimal supplementation of growth factors. With the murine angiogenesis model of subcutaneous matrigel implantation, we confirmed that platelet releasates promote angiogenesis in vivo, and that the enghancement by PAR1-PR was more marked than that by PAR4-PR (figure 6). The latter was seen as more intense formation of new blood vessels and a denser network of blood vessels.

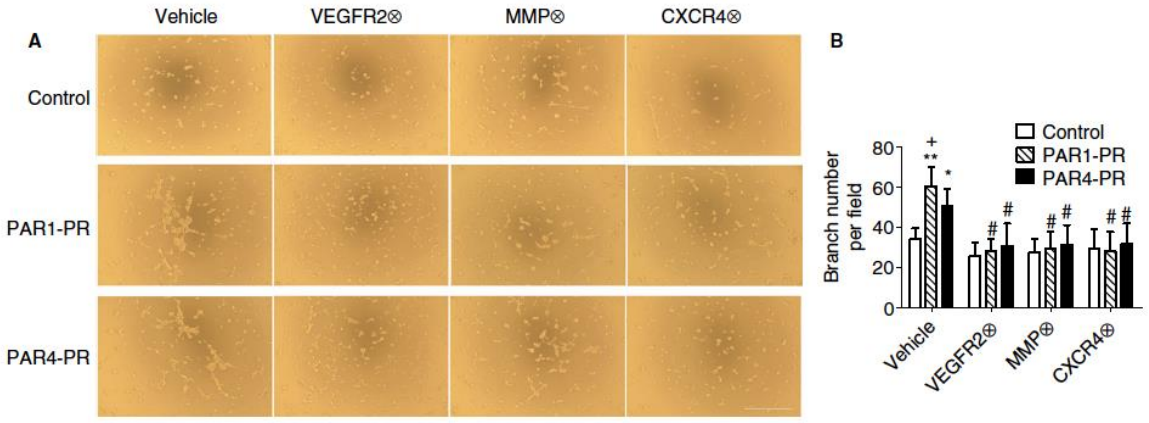


***Figure 5*. PAR1-PR surpasses PAR4-PR in promoting tube formation of EPCs in basal culture medium, while intervention of VEGF, MMP or SDF-1$\alpha$ inhibits platelet releasate-enhanced EPC tube formation.**

Some early studies have shown that thrombin receptor PAR1 stimulation selectively evokes platelet release of proangiogenic regulators, while PAR4 stimulation is prone to selective release of antiangiogenic regulators [104, 105, 115], albeit there are still debates about the theory [106, 107]. Hence, our original hypothesis was that PAR1-PR would promote angiogenesis, while PAR4-PR might attenuate angiogenesis of EPCs. However, we were unable to prove our hypothesis in the present study. Instead, we found that both PAR1-PR and PAR4-PR had stimulatory effects on angiogenic activities of EPCs, and that angiogenesis-stimulatory effects of PAR1-PR was more potent than those of PAR4-PR. One possible explanation may be that the selective release of platelet angiogenic regulators mainly concerns the different release levels of platelet angiogenic regulators, and that the absolutely selective release was seen only with endostatin secretion triggered by PAR4 stimulation. On the other hand, platelets store and release many different angiogenic regulators. The final outcome of angiogenic regulating effects will depend on the negotiation of all factors in the platelet releasates.

The concept of different platelet stimulus-induced distinct releases of platelet proangiogenic and antiangiogenic regulators has been independently proved at several laboratories [105,

106, 116]. Our data have provided a new support to the concept. Moreover, there is new evidence suggesting that the distinct release may be activation intensity dependent, because the difference in platelet releasates may diminish on intensifying platelet activation by prolonged stimulation [117] or extra-high concentrations of PAR1 and PAR4 agonists [108].

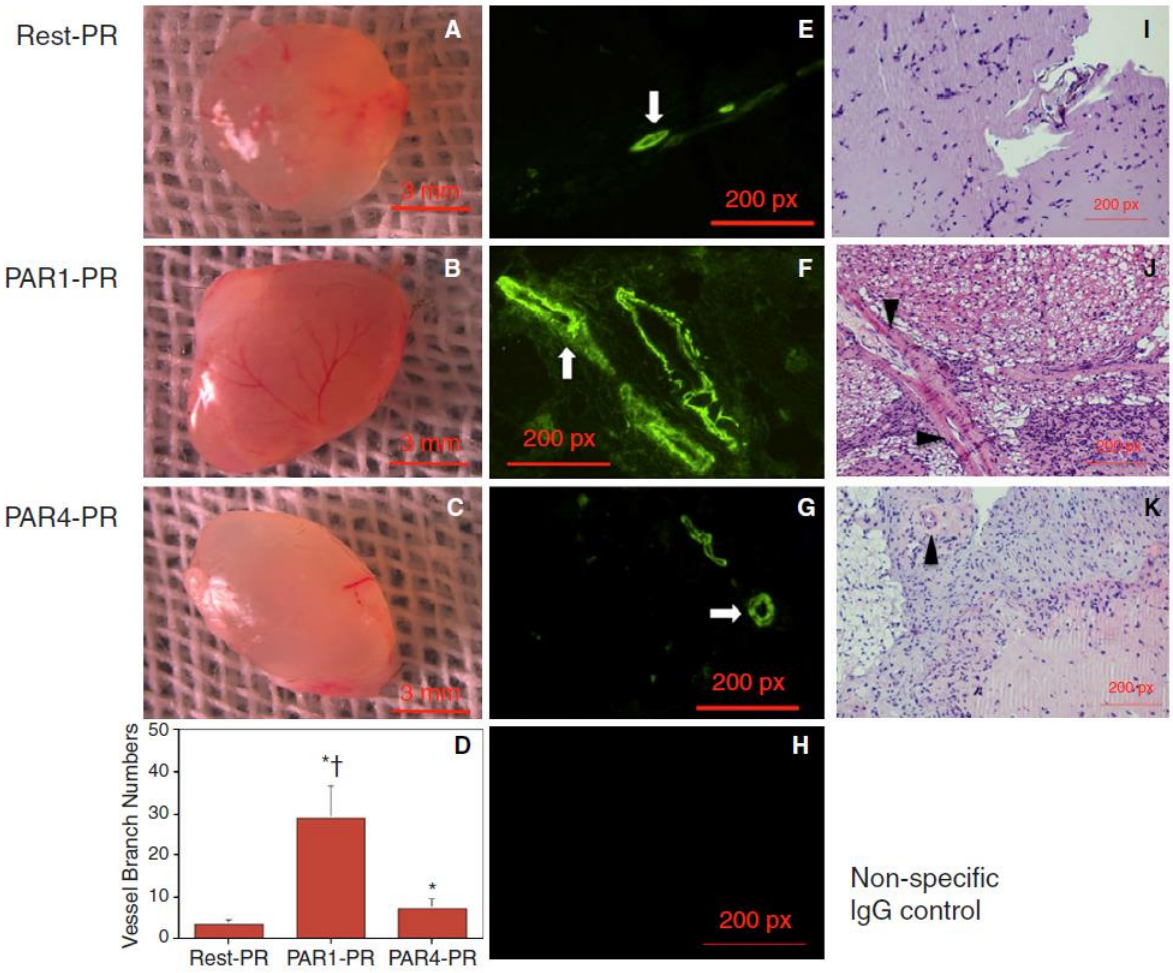


*Figure 6.* **PAR1-PR promotes stronger vasculogenesis in vivo than PAR4-PR.**

## 4.2 TETRASPANIN CD151 AND INTEGRIN α6β1 MEDIATE PLATELET ENHANCED ENDOTHELIAL PROGENITOR CELL ANGIOGENESIS

### 4.2.1 EPC angiogenic responses induced by platelets were stronger than those induced by platelet releasates

In Paper I, we have shown that platelet releasate can enhance EPC angiogenesis. In Paper II, we first showed that platelets cell-concentration-dependently enhanced tube formation of EPCs (referred as endothelial colony forming cells, ECFCs, in Paper II). Afterwards, we compared the effects of whole platelets and total platelet releasate on EPC tube formation with an in vitro Matrigel model. It was found that, albeit both platelets and total platelet releasate enhanced EPC tube formation, the angiogenic responses to platelets were more pronounced than those to total platelet releasate, and that higher increases of the branch

points of EPC tube formation in the presence of whole platelets (Figure 7). The stronger enhancements by whole platelets indicate that platelet components other than platelet-released mediators contribute to the enhancements.

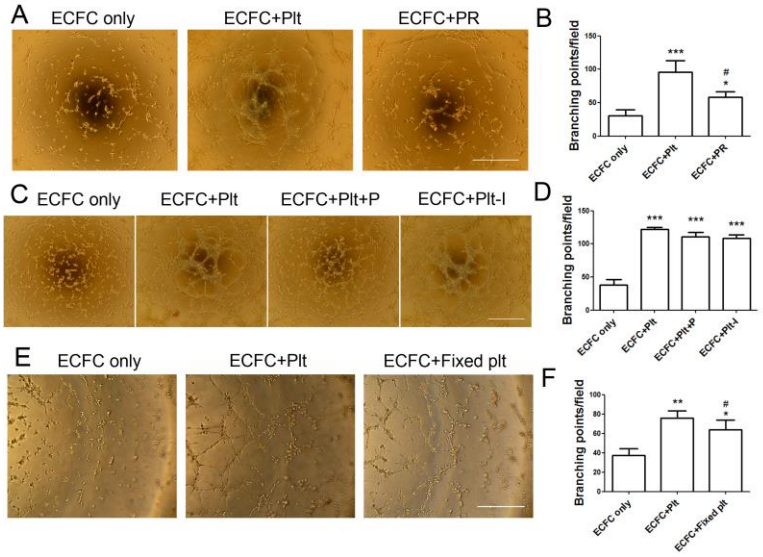


*Figure 7.* **Platelets surpass platelet releasate in promoting tube formation of EPCs in basic culture medium.**

### 4.2.2 Platelet membrane components contribute importantly to the platelet-enhanced EPC tube formation

We have performed a series of experiments to figure out platelet components contributing to the enhancements. Thus, platelets treated with puromycin, which blocks de novo protein synthesis of platelet angiogenic regulators, had no effect on platelet-enhanced EPC tube formation, suggesting that de novo protein synthesis did not contribute importantly to the enhancement in the present setting. Similar results were alos found in platelet-enhanced EPC tube formation in the presence of Iloprost, which irreversibly inhibit platelet activation and secretion. Furthermore, 2% paraformaldehyde-fixed platelets had only slightly weaker enhancement than unfixed platelets. All these results suggest that the physical presence of platelets is necessary for an optimal platelet-enhanced EPC tube formation, and that platelet membrane components can contribute importantly to the process.

### 4.2.3 Platelet CD151 and Integrin α6β1 is involved in platelet-enhanced EPC tube formation

Platelets pretreated with neuraminidase, which strips sialic acid residues from glycoproteins, failed to promote tube formation. The results suggest that platelet surface glycoproteins have a major role in promoting EPPC tube formation. In order to identify the platelet membrane proteins responsible for the platelet pro-angiogenic effects, we employed a panel of blocking agents for platelet membrane components, it was found that platelet CD151 blockade, but not CD151 blockade on EPCs, significantly attenuated platelet-enhanced EPC tube formation (Figure 8).

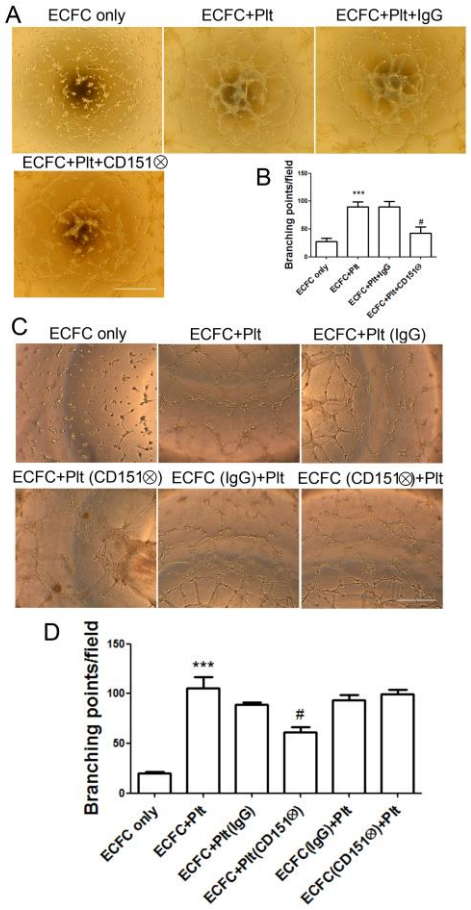


***Figure 8*. CD151 on platelets but not on EPCs is important for platelet-induced tube formation of EPCs.**

CD151, together with other tetraspanins, is known to organize membrane microdomains [118]. Tetraspanins interact with other membrane-embedded proteins to form functional clusters of proteins and to regulate multiple cellular processes. We have demonstrated in the present study that α6β1 integrin is the partner for platelet CD151 in platelet CD151-mediated enhancement of EPC capillary network formation, as evidenced by the marked inhibition of EPC tube formation upon α6β1 integrin blockade. It is well known that α6β1 is a laminin - binding integrin. It may interact with laminin-111 presented in matrigel and thereby mediate angiogenesis, as recently demonstrated by Bouvard et al [119]. Moreover, we showed that Src kinase signaling is critical in platelet-enhanced angiogenesis of EPCs, because Src inhibition abolished the enhancement, and that PI3K signaling is also involved, as PI3K inhibition partially reduced platelet-enhanced ECFC tube formation. Because Src is a key node of α6β1 outside-in signaling [120], our data support the notion that platelet CD151 mediates platelet-enhanced EPC tube formation via regulating α6β1-lamimin interaction and through Src-mediated α6β1 outside-in signaling.

## 4.3 THROMBIN-REDUCED PLATELET MIR-27B EXPRESSION ENHANCES DE NOVO SYNTHESIS OF THROMBOSPONDIN-1 IN PLATELETS

### 4.3.1 miR-27b was down-regulated in platelet upon thrombin stimulation

Microarray-based miRNA profiling was done in both total platelet RNAs and AGO2-pulled down RNAs. With SAM analyses, we found that 9 miRNAs were over-expressed, and that 12 miRNAs were under-expressed in total platelet lysate. In contrast, we found that 26 miRNAs were down-regulated but no miRNAs were up-regulated in Ago2 associated-miRNAs. Interestingly, only miR-27b-3p was identified overlapping between these two groups of altered platelet miroRNAs. The data of qRT-PCR experiments verified that the expression levels of miR-27b-3p were significantly reduced in platelets after thrombin stimulation. The result is a clear sign that platelet miRNAs may exert regulatory roles in platelets *per se*. Moreover, all down-regulated Ago2-associated miRNAs imply that platelet miRNAs likely enhance protein synthesis of activated platelets.

### 4.3.2 TSP1 was up-regulated after thrombin stimulation in platelets at both mRNA level and protein level

TSP-1 is a potent endogenous inhibitor of angiogenesis, through direct effects on endothelial cell migration, proliferation, survival, and apoptosis, and indirectly by antagonizing the activity of VEGF. Moreover, the genome-wide RNA-seq analysis of human platelet transcriptomes has shown that TSP1 mRNA exists in quiescent platelets [91]. Hence, it would be interesting to investigate if TSP1 mRNA can be up-regulated upon thrombin stimulation. Our qPCR results showed that TSP1 mRNA significantly increased after thrombin stimulation, indicating a platelet activation-dependent splicing of TSP1 pre-mRNA. TSP1 western blot demonstrated that thrombin-induced platelet activation decreased platelet TSP1 content, indicating a significant release of TSP1 upon platelet activation, and that the decreased TSP1 levels were not only fully recovered but also markedly elevated after 24 h culture. The latter indicates that thrombin stimulation induced a marked de novo synthesis of TSP1 in platelets. As a well-established anti-angiogenic regulator, the marked de novo synthesis of TSP1 in activated platelets may be an important negative regulatory mechanism of platelet angiogenic activities.

### 4.3.3 miR-27b mimic can down-regulate TSP1 expression in MEG-01 cells

It has been previously shown that miR-27b can regulate TSP1 synthesis [121].We showed in the present study that miR-27b was associated with platelet Ago2 complex, and was reduced upon thrombin stimulation. Because thrombin stimulation induced platelet TSP-1 mRNA maturation and a marked surge of platelet TSP1 content, we hypothesized that miR-27b may be the regulator for the dramatic de novo protein synthesis of TSP1 thrombin-activated platelets. These associated expression indicated that de novo synthesis of TSP-1 maybe regulated by miR-27b in platelets. To prove our hypothesis, we chose to transfect miR-27b mimics into the megakaryocyte cell line MEG-01 cells, as platelets are known for their low transfection rate using an ordinary transfection protocol. Our result (Figure 9) showed that

TSP1 protein expression was down-regulated by miR-27b mimics, but not by miR-27b mimics control, transfection. Therefore, the present work has provided the first evidence that platelet miRNAs can regulate platelet protein synthesis.

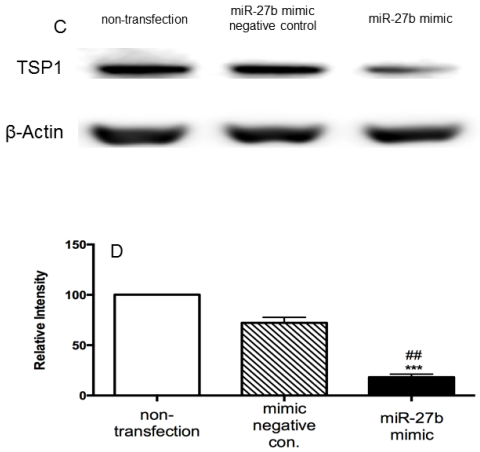


***Figure 9.*** **miR-27b mimic reduces TSP-1 protein synthesis of MEG-01 cells.**

## 4.4 UNALTERED ANGIOGENESIS-REGULATING ACTIVITIES OF PLATELETS IN MILD TYPE 2 DIABETES MELLITUS (T2DM) DESPITE A MARKED PLATELET HYPERREACTIVITY

### 4.4.1 Platelet hyperactivity in T2DM patients

The basal level of platelet P-selectin expression in whole blood was similar between T2DM and non-diabetic subjects as assessed by flow cytometry. However, upon stimulation, platelet P-selectin expression increased more markedly in T2DM patients than in non-diabetic controls. Similarly, PAR1-AP enhanced platelet phosphotidylserine (PS) exposure/annexin V binding much more marked and rapid in T2DM patients. Thus, more than 75% increment of annexin V binding had already been reached by 2 min in T2DM patients, as compared to that of less than 50% in the control subjects. The stimulus also induced a greater increase of annexin V binding in T2DM patients (30.9±5.1% at 8 min) than in the controls (24.3±3.0%; $P<0.01$). Our work brought new evidence showing that diabetic platelets had a more rapid and more intense exposure of aminophospholipids (predominantly phosphotidylserine), which is a key component of platelet procoagulant activities and constitutes the prothrombinase complex together with FXa, FVa, and calcium. The alteration is likely a key element contributing to the hypercoagulant status in T2DM patients.

### 4.4.2 Platelet release of angiogenic regulators in T2DM and control subjects were similarly enhanced by PAR1-AP and PAR4-AP

Activated platelets release a number of angiogenic regulators, which can regulate EPC (referred as ECFC in Paper IV) tube formation. We assumed that altered platelet reactivities in T2DM patients might lead to an altered release pattern of platelet angiogenic regulators. Using a set of ELISAs for quantifying platelet-released VEGF, PDGF, PF4 and TSP-1, we found that PAR1 and PAR4 stimulation induced similar releases of pro- or anti-angiogenic

regulators in T2DM and control subjects. Our hypothesis was thus not proven, at least in the present group patients with mild or well controlled T2DM.

### 4.4.3 The enhancements of EPC tube formation by platelet releasates from T2DM patients were similar to those from non-diabetic controls

The effects of platelet releasates on EPC tube formation were examined with an in vitro Matrigel model, with the supplementation (10%) of either PAR1-PR or PAR4-PR from either control subjects or T2DM patients. As expected, both PAR1-PR and PAR4-PR enhanced tube formation of EPCs, and the enhancements by PAR1-PR and PAR4-PR from T2DM patients were similar to those from non-diabetic controls.

### 4.4.4 Platelet-enhanced EPC tube formation did not differ between non-diabetic and diabetic subjects

To investigate if there is an alteration of platelet contact-dependent enhancement of EPC angiogenic activities in T2DM patients, platelets were isolated from patients and controls, and co-incubated with EPCs on a Matrigel-coated plate to monitor their effects on the capillary network formation. The presence of platelets from either control or T2DM subjects markedly enhanced EPC tube formation, but the enhancements did not differ between non-diabetic and diabetic platelets (Figure 10).

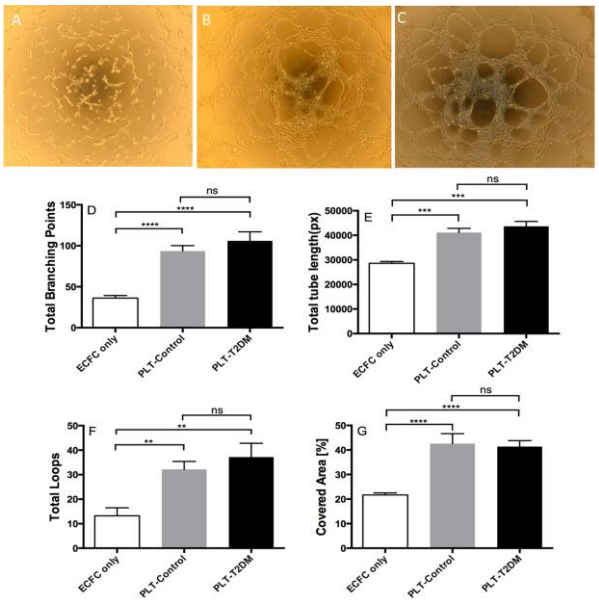


***Figure 10.*** **Diabetic and non-diabetic platelets similarly enhance tube formation of EPCs.**

Together, our results showed that 1) platelet release of angiogenic regulators were similar in the mild diabetic and non-diabetic subjects, 2) that supplementation of platelet releasates from patients and controls had identical effects on EPC tube formation, and 3) non-diabetic and diabetic platelet co-culture similarly enhanced EPC tube formation. These three pieces of evidence indicate that platelet α-granule contents/release of angiogenic regulators and platelet membrane components involved in platelet-enhanced angiogenesis (e.g., CD151) are unaltered in well controlled/mild T2DM patients. Hence, one may assume that disturbed

platelet angiogenic activities would more likely be seen in advanced/poorly controlled T2DM patients, and that the dysfunctions might happens secondary to a poor blood glucose control. As blood glucose and HbA1c of the present T2DM patients were controlled on decent levels, our data may suggest that a good glucose control is beneficial for maintaining platelet angiogenic function, despite the presence of diabetic platelet hyperreactivities.

# 5 SUMMARY AND CONCLUSIONS

Platelets are an active engager in angiogenesis. The present thesis work provides several pieces of new evidence showing that platelet angiogenic activities are a concert of multiple players:

1. The platelet releasates from PAR1- and PAR4-stimulated platelets enhanced tube formation of EPCs both in vitro and in vivo, but had no effects on EPC proliferation. Albeit PAR1 and PAR4 stimulation may lead to selective releases of pro- and anti-angiogenic regulators, the total impact of PAR1-PR and PAR4-PR on angiogenesis remain stimulatory, while the angiogenesis-stimulatory effects of PAR1-PR are more potent than those of PAR4-PR.

2. Besides platelet-released mediators, platelet membrane components can promote EPC angiogenesis. Thus, platelet membrane-expressed tetraspanin CD151 regulates EPCs angiogenesis through interactions with α6β1 integrin. The optimal enhancement of EPC angiogenesis by platelets requires both membrane proteins and platelet-released angiogenic regulators. Therefore, in addition to platelet-released angiogenic factors, platelet membrane components may serve as a useful target for intervention of platelet angiogenic activities.

3. Thrombin stimulation induces marked de novo synthesis of TSP-1 (a well known anti-angiogenetic factor) in platelets, which is associated with thrombin-induced down-regulation of Ago2-associated miR-27b. Using the megarkaryocyte cell line Meg-01 cells and miR-27b mimics transfection, we have demonstrated that down-regulated miR-27b may be a key regulator of de novo protein synthesis of TSP1. Therefore, the present work has provided the first evidence demonstrating that platelet miRNAs can regulate protein synthesis in platelets per se.

4. Platelets are hyperreactive in T2DM patients. However, platelet angiogenic activities, both in terms of platelet release of angiogenic regulators and platelet membrane component-dependent angiogenic activity, remain unaltered in well controlled/mild T2DM subjects. The finding suggests that a good glucose control may be beneficial for maintaining platelet angiogenic function in the patients.

# 6 FUTURE PERSPECTIVES

Platelets are well recognized in their engagements in angiogenesis. Platelets are an active player in angiogenesis in many physiological and pathophysiological processes, such as embryo development and growth, tissue regeneration, vessel remodelling, as well as tumour growth and metastasis. The present thesis work has provided new evidence demonstrating a complex engagement of platelets in angiogenesis. The work has shown that platelets enhance angiogenesis not only through platelet-released angiogenic regulators but also through direct cell-cell contact via membrane components (including the tetraspanin CD151 and the integrin α6β1). Moreover, the thesis work has also shown that down-regulated platelet mi-R27b may contribute to the surge of de novo protein synthesis of TSP1 in activated platelets, and subsequently regulate platelet angiogenesis. Our findings support the notion that platelet angiogenic activities are a concert of multiple players. Of course, our work also raises new issues to be addressed in our future studies.

There is still a debate with regard to the concept of distinct packaging and differential release of platelet pro- and anti-angiogenic regulators upon stimulation of different platelet agonists. It is, however, general accepted that there is an activation intensity-dependent selective release of platelet pro- or anti-angiogenic regulators. Therefore, it is of interest to elucidate the intracellular signaling mechanisms governing the selective release of platelet pro- or anti-angiogenic regulators.

Platelet membrane components, the tetraspanin CD151 and the integrin α6β1, contribute importantly to platelet-enhanced angiogenesis. It is tempting to ask if other tetraspanins, such as CD9, which is highly expressed on platelets, also contribute to platelet angiogenic activities. Moreover, tetraspanins are known as an organizer of membrane proteins. The mechanism with regard to how platelet CD151 "organizes" α6β1 on platelet membrane, and then transfer the signal to ECs/EPCs for an enhanced angiogenesis remains to be elucidated.

Platelets are rich in miRNAs, however, their function in platelet biology remains unclear. The present thesis work showed that thrombin stimulation induced alteration of miRNAs profile in platelets. However, it is difficult to demonstrate the functional outcomes of these altered miRNAs in platelet-associated angiogenesis, because mature platelets can hardly be transfected using ordinary transfection kits/protocols. There is a need to establish an effective system of platelet transfection.

Platelet activation is a complicated process, and can be triggered by various stimuli. It is of interest to investigate the alteration of miRNAs in platelets upon other agonists, such as ADP, collagen and $TXA_2$. The work may help to identify new intervention strategies targeting miRNAs for the development of novel anti-angiogenesis and anti-thrombotic drugs.

Our results have shown unaltered platelet angiogenic activities in mild and well-controlled type 2 diabetes mellitus patients, despite clear signs of platelet hyperreactivities. Hence, it is desirable to investigate platelets angiogenic function in advanced and/or poorly-controlled

type 2 diabetes mellitus patients. Moreover, it should also be interesting to study if optimization of anti-diabetic treatments could improve platelet angiogenic activities.

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